Universidade de Lisboa

Faculdade de Farmácia

Nanoparticles as carrier systems for protein delivery

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Resumo

As terapias com recurso a proteínas têm apresentado um desenvolvimento significativo ao longo das últimas décadas, constituindo novas opções terapêuticas para um grande número de doenças. Contudo, a entrega bem sucedida das proteínas continua a ser uma tarefa difícil, uma vez que estas podem sofrer degradação enzimática na circulação sistémica, apresentam baixa permeabilidade celular e, consequentemente, biodisponibilidade reduzida, limitando a sua aplicação. Nesta revisão da literatura são revisitados conceitos-chave na área da nanomedicina, bem como várias abordagens desenvolvidas para o transporte e entrega de péptidos e proteínas.

Os nanotransportadores são especificamente desenhados para proteger os fármacos da biodegradação, controlar a sua libertação, permitir atingir de forma eficiente os orgãos e tecidos alvo e reduzir a citotoxicidade. Um nanotransportador ideal deve ser biocompatível e biodegradável, apresentar uma eficiência de encapsulação elevada e uma grande capacidade de manter a estrutura e a actividade da proteína. Para além disso, a sua produção deve ser simples e reprodutível, deve apresentar opções de administração clinicamente relevantes e ser economicamente viável. Propriedades como o tamanho, a forma e a superfície devem ser tidas em conta no desenvolvimento de novos nanotransportadores, dado que têm um papel fundamental na estabilidade, especificidade em relação ao alvo e cinética de libertação dos fármacos, que são aspectos determinantes para a sua eficiência.

Existem vários tipos de nanotransportadores, quer orgânicos quer inorgânicos, incluindo nanopartículas de lípidos sólidos, lipossomas, nanoparticulas de polímeros, nanopartículas víricas, nanopartículas de sílica mesoporosa, nanopartículas metálicas e nanopartículas magnéticas. A toxicidade destas particulas é altamente determinada pelas suas propriedades físico-químicas, uma vez que estas influenciam a forma como as partículas interagem com as células. O conhecimento destas interaçóes permite o desenvolvimento de nanopartículas mais seguras.

Foram desenvolvidos e introduzidos na prática clínica vários nanomedicamentos e existem muitos outros que se encontram ainda em fase de investigação. No entanto, os numerosos problemas técnicos, associados à falta de protocolos padrão para a caraterização fisico-química e fisiológica/biológica de novas formulações, têm comprometido o desenvolvimento e aprovação de diversas terapias. Apesar de todos os problemas que ainda necessitam de resolução, as nanopartículas com proteínas constituem uma grande promessa como agentes terapêuticos, aumentando a biodisponibilidade e controlando a libertação das proteínas, ao mesmo tempo que as direccionam de forma eficiente para os órgãos e tecidos alvo.

Palavras-chave: nanotransportadores, nanoterapias, nanopartículas, proteina, entrega.

Abstract

Protein-based therapies have significantly developed over the past decades, providing new therapeutic options for a wide range of diseases. However, successful protein delivery remains a challenging task, since they can be degraded by enzymes in systemic circulation, present low cell permeability and have poor bioavailability, thereby limiting their clinical application. This review revisits the fundamental concepts in the field of nanomedicine, as well as several approaches developed for peptide and protein delivery.

Engineered nanocarriers are specifically designed to protect drugs from biodegradation, control their release and clearance, and allow efficient targeting of organs and tissues, with reduced cytotoxicity. An ideal nanocarrier must show biocompatibility, biodegradability, elevated encapsulation efficiency, high capacity to keep protein structure and bioactivity, simple and reproducible production, clinically relevant administration options, and economic feasibility. Properties such as size, shape and surface must be considered in the design of a new nanocarrier, as they play a significant role in the nanoparticles' stability, targeting specificity and drug release kinetics, thus directly affecting their therapeutic efficacy.

There are a vast number of nanocarrier's types from organic to inorganic structures, including solid lipid nanoparticles, liposomes, polymeric nanoparticles, virus-based nanoparticles, mesoporous silica nanoparticles, metallic nanoparticles and magnetic nanoparticles. The toxicity of these particles is highly determined by their physical and chemical properties, since they influence how the particles interact with cells. Thus, understanding these interactions can lead to the development of safer nanoparticles.

Several nanomedicines have been developed and commercially approved for clinical use, with many more being currently under clinical investigation. However, the numerous technical issues coupled with the lack of standard protocols for physicochemical and physiological/biological characterization of new formulations have compromised the development and approval of many therapies. Despite all the issues that still need to be addressed, protein-loaded nanoparticles hold great promise as new therapeutic agents for targeted therapies, increasing protein bioavailability, controlling their release and efficiently targeting organs and tissues.

Keywords: nanocarriers, nanotherapies, nanoparticles, protein, delivery.

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List of Abbreviations

ABC	Accelerated blood clearance
ADA	Adenosine deaminase
AgCl	Silver chloride
AgI	Silver iodide
AgNO3	Silver nitrate
AgNPs	Silver nanoparticles
ALP	Alkaline phosphatase
AuNPs	Gold nanoparticles
BBB	Blood brain barrier
BMP-2	Bone morpgogenetic protein-2
BMSCs	Bone mesenchymal stem cells
BUN	Blood urea nitrogen
CaPNPs	Calcium phosphate nanoparticles
CKD	Chronic kidney disease
CNTF	Ciliary neurotrofic factor
CNTs	Carbon nanotubes
CS-TPP NPs	Chitosan-tripolyphosphate nanoparticles
DEX	Dexamethasone
EEA	European Economic Area
EMA	European Medicines Agency
EPO	Erythropoietin
EPR	Enhanced permeability and retention
FDA	US Food and Drug Administration
GI	Gastrointestinal
HA	Hyaluronic acid
HPH	High-pressure homogenization
HPMCP	Hydroxypropyl methylcellulose
IFN- α2a	Interferon- α2a
IFN- α2b	Interferon- α2b
IgAN	Immunoglobulin A nephropathy
MNPs	Metallic nanoparticles
MRI	Magnetic resonance imaging

MSNs	Mesoporous silica nanoparticles
NPs	Nanoparticles
PCCs-NPs	Phosphorylcholine-chitosan nanoparticles
PEG	Polyethylene glycol
PGA	Poly(glycolic acid)
PLGA	Poly(lactic-co-glycolic acid)
PLA	Polylactide
PNPs	Polymeric nanoparticles
QDs	Quantum dots
RBCs	Red blood cells
RES	Reticuloendothelial system
ROS	Reactive oxigen species
SCID	Severe combined immunodeficiency
SH3	Src homology 3
SLNs	Solid lipid nanoparticles
TiO_2	Titanum dioxide
VLNP	Virus-like nanoparticles
VNPs	Viral nanoparticles

I. Introduction

Peptides and proteins are dynamic and versatile macromolecules which are able to perform a complex and unique set of functions, playing a major role in living systems.(1)(2) They are involved in diverse intracellular processes, including enzyme catalysis, signal transduction, gene regulation and maintenance of the balance between cell survival and programmed death.(2) Along with the ability to perform a variety of complex functions, proteins have low tendency to disrupt biological processes, making them suitable candidates for several biomedical applications.(2)(3)

Protein-based therapies have significantly developed over the past decades, from fully human antibodies to chimeric proteins and new scaffolds capable of binding to undruggable targets, providing new therapeutic options for a wide range of disease states, such as cancer, diabetes, lysosomal storage and transient cerebrovascular disorders, infection, and inflammation.(3)(4) Therapeutic approaches using peptides and proteins have noteworthy advantages over many conventional therapies, including greater effectiveness, higher specificity, better activity, and less toxicity.(4)(5) Intracellular delivery of functional proteins can replace missing, dysfunctional, or poorly expressed endogenous proteins or even antagonize key pathways that occur inside the cell.(3) Despite all these advantages, successful protein delivery remains a challenging task, since they can be degraded by enzymes in systemic circulation, present low cell permeability and have poor bioavailability, thus limiting their clinical application.(5)

Proteins are tertiary molecules, which make them more susceptible to attacks or physical and chemical changes in their surrounding environment, resulting in structural damage and, consequently, impaired function.(2) Not only can this sensitivity be associated to the development of several diseases, but it is also a limitation to the clinical use of proteins, since many physiologic processes such as hydrolysis, oxidation and proteolysis can induce structural damages too, making it difficult to deliver the unmodified functional protein in an active conformation to the site of action.(1)(2)(3) Due to their instability, protein drugs are traditionally administered by intravenous injection rather than taken orally like most small chemical drugs.(6) Orally administered proteins are not efficiently delivered into the bloodstream, as they are easily degraded in the stomach by an acid catalyzed process and can undergo proteolytic breakdown throughout the gastrointestinal tract. Moreover, their permeability across gastrointestinal mucosa is poor and they are susceptible to being eliminated during first-pass metabolism in the liver.(2)(6)

Parenteral delivery can avoid biological barriers. However, protein and peptide drugs usually have *in vivo* half-lives in the range of a few minutes to a few hours following systemic administration.(7) Furthermore, their high molecular weight, surface polarity and

immunogenicity also difficult their delivery into the cell.(8)(9) Therefore, potential therapeutic proteins often require modification, encapsulation or immobilization with biocompatible matrices, in order to improve their stability, activity, immunogenicity, and delivery.(1) Another advantage of these techniques is the reduction of the total concentration required to obtain therapeutic benefits, thus decreasing the cost of the therapy. Like other strategies, these protein carriers have significant limitations, including low encapsulation efficiency, physical instability, toxicity to cells or tissues and activity reduction due to harsh manufacturing conditions or undesirable degradation products.(1)(2) Moreover, the high specificity of proteins often requires maintaining their structural complexity, which can make them difficult to modify and/or formulate.(4)

The development of novel methods for peptide and protein administration is a complex task, that requires the combination of an optimal administration route with chemical modification of amino acids, in order to increase the stability of the molecules, and thus enhance their bioavailability.(10) The encapsulation of proteins in micro and nanoparticles (NPs) has gathered wide notability due to their broad application potential as biosensors or bioreactors. Hence, extensive research efforts have been made towards finding and characterizing suitable protein delivery carriers.(5)(11) Engineered nanocarriers are specifically designed to protect drugs from biodegradation, control their release and clearance, and allow efficient targeting of organs and tissues, with reduced cytotoxicity. It is also possible to load multiple drugs simultaneously, enabling them to act in a synergic manner.(10)(12)

Despite the significant progresses made in the last few decades, several challenges still need to be addressed. The aim of this review is to revisit some of the fundamental concepts in the field of nanomedicine, as well as to discuss the state of the art of nanotherapeutics using protein-based drugs.

II. Methods

The research work for this review started with the search for fundamental concepts in the field of nanomedicine, more specifically in what concerns to nanoparticle characterization. This summary was followed by a more refined research focused on the use of NPs for peptide and protein delivery, where both investigational and currently approved protein-based nanotherapies were analyzed.

The articles cited in this review were gathered between February and August of 2019, through web-based searches of main databases, including PubMed, Science Direct and Google Scholar. Websites from reference entities, such as US Food and Drug Administration (FDA), European Medicines Agency (EMA) and INFARMED were also assessed. Searches included different words with the prefix nano-, including ‘nanocarriers(s)’, ‘nanoparticle(s)’, ‘nanosystem(s)’ and ‘nanotherapy(ies)’ combined with terms like ‘protein’, ‘drug’ and ‘delivery’ in order to refine them while further focusing and limiting the selection. In parallel, reference scanning was used to identify other studies that have shown to be relevant for the full comprehension of the subject.

III. Results

1. Ideal carrier system

As aforementioned, loading proteins in a delivery system has several advantages over soluble formulations. An ideal protein or peptide delivery system should address a set of requirements, including safety and biocompatibility, biodegradability, elevated encapsulation efficiency, high capacity to keep protein structure and bioactivity, simple and reproducible production, clinically relevant administration options, and economic feasibility. Many applications also require controlled release, long circulation half-life, intracellular delivery and targeting ability.(1)(13) These systems are very similar to biological entities, such as viruses, and are especially needed when the therapeutics to be delivered require specific handling.(10)(14)

2. Design

Nanocarriers are not drugs themselves, but can be loaded with drugs, genes, antibodies, or radioactive materials, and their surface can be functionalized in order to direct them to exert their activity on a specific site.(15) To design a new carrier, properties such as size, shape and surface must be considered as they play a significant role in the NPs stability, targeting specificity and drug release kinetics, thus directly affecting their therapeutic efficacy.(2)

2.1. Size and shape

NPs typically have a diameter range from 1 to 100 nm.(14) Particle size is a crucial parameter that directly determines the surface area available to interact with biological environments, thus affecting the efficiency of drug delivery to various parts of the body.(14)(16)(17) Besides, size plays a critical role in the accumulation and penetration of nanocarriers at the disease sites.(17) In normal blood vessels, the smooth muscle layer is essential for mediating vasogenic response to vascular mediators and, hence, for maintaining a constant blood flow to an organ.(18) Conversely, the microvasculature of inflamed or neoplastic tissues lacks these smooth muscle cells, as a result of deregulated angiogenesis and/or increased expression and activation of vascular permeability factors.(18)(19) This imbalance, described for the first time by Maeda et al.(20) in 1986, is called the enhanced permeability and retention (EPR) effect, and results in a discontinuous endothelial layer, where fenestrations between the endothelial cells may range from 300 to 4700 nm, allowing the extravasation of large molecules and particles.(19) Such enhanced permeation leads to increased accumulation of NPs in these tissues, when compared to other organs. (21) However, it is important to refer that in many pathological conditions the integrity of vascular endothelium remains unaffected and there is no opportunity for EPR.(22)

Last of all, size significantly influences blood circulation time and biodistribution of nanocarriers.(17) Generally, particles with a diameter range from 10 to 200 nm remain stable in the bloodstream, which makes them more likely to reach and accumulate on the inflammation/tumor sites. On the other hand, particles with larger dimensions will be preferentially captured by the reticuloendothelial system (RES) and rapidly cleared from circulation; whereas particles lower than 5 nm would be easily eliminated by renal filtration.(23) Nonspecific targeting mechanisms rely essentially on this phenomenon.(24)

The shape of nanocarriers is another key feature which determines blood circulation time and vessel wall adhesion.(25) Both size and shape of particles are likely to influence particle transport behavior in the blood, especially in small capillaries and tumor vasculatures, as well as how cells sense and react to the particle endocytosis. Thus, circulation time, targeting, and the ability to overcome biological barriers could depend on this properties.(26) Besides, geometry affects surface to volume ratio, so shape is also likely to influence *in vivo* biodistribution, pharmacokinetics, and degradation of the drugs, compromising their ability to target certain sites.(24)(26) Worm-like particles with high aspect ratios, i.e. particles which have a length many times greater than their width, showed negligible phagocytosis, when compared to conventional spherical particles of equal volume.(27)

To be effective, a nanocarrier should be able to interact in an efficient manner with the capillary wall and “migrate” to the target tissue before being cleared by the RES or being filtered by the lungs, liver, and spleen.(26) Migration of NPs towards blood vessel walls – margination - is a crucial step for a successful delivery of the drug to the target site, since the interaction between particles and the microvasculature is required. Thus, particles can either target disease-specific vascular biomarkers or extravasate through the leaky endothelium into the interstitial space.(28)(29) Margination strongly depends on the distribution of the carriers within vessel cross-sections. Among other parameters, including blood flow properties and vessel size, nanoparticle distribution is affected by particle size, shape and deformability. Particle margination is mediated by the migration of red blood cells (RBCs) to the vessel center, as a result of the hydrodynamic interactions with the walls – lift forces – creating a RBC-free layer near the walls.(29) Due to the balance of forces acting on nanocarriers, including hydrodynamic drag, van der Waals and steric interactions, particles with size of about 100 nm are not suitable for drug delivery, since they show a tendency to stay away from the endothelium. Particles smaller or larger than this size tend to experience margination, which makes them more advantageous for delivery applications.(30)

Using an *in vitro* model, Toy et al.(28) evaluated the effects of particle shape, size and density on NPs’ margination. The results showed that smaller-sized and oblate-shaped particles have higher margination rates. Furthermore, lighter particles are more likely to undergo margination.

Müller and colleagues(29) also investigated the role of particle size and shape on the margination efficiency, employing mesoscopic hydrodynamic simulations of the blood flow. The simulations demonstrated that the greater the size of the carrier the greater its margination potential, contradicting the previous authors. Concerning shape, although spherical particles yield slightly better margination, ellipsoidal particles exhibit slower rotational dynamics near a wall, which favors their adhesion.

2.2. Surface functionalization and targeting mechanisms

Surface properties of NPs, such as surface charge, surface hydrophobicity and targeting ligands, are particularly important for a successful delivery of the drug, since they directly determine the interactions with the biological microenvironment, influencing biodistribution, cellular uptake, immune system activation, and the composition of the so-called protein corona that develops around NPs *in vivo*.(17)(31)(32) In this manner, surface functionalization through controlled chemical modifications is an essential tool to modulate NPs' *in vivo* behavior.(33)

Generally, positive-charged NPs easily bind to the cell membrane, which has an intrinsic negative surface charge. However, this property might also strengthen their nonspecific binding to normal tissues.(34) Moreover, endothelial cells of blood vessels also exhibit a negative charge, due to the anionic glycocalyx layer, which not only establishes a "charge barrier" that repels the attachment of negatively charged blood cells and plasma molecules, but also attracts NPs with high positive charges, which will bind nonspecifically to the luminal surface of the vascular walls and be rapidly cleared from the blood circulation.(34)(35) Another effect to be taken into account is the non-specific adsorption of proteins over the NPs' surface, in which the surface charge has significant implications, influencing the species of adsorbed plasma proteins.(17)(36) Surface charge is also highly responsible for the targeted accumulation of NPs in the disease sites.(17)

NPs, like pathogens, are subject to the body's immune response, activating both innate and adaptive immune mechanisms.(37)(38) Surface hydrophobicity plays a key role in immune system activation by inducing opsonization.(17) Once NPs reach blood circulation, they interact with plasma proteins, which bind to their surface forming the protein corona. This process promotes the binding of immunoglobulins to the nanoparticle's surface, enabling its recognition and uptake by the phagocytic cells.(38) NPs with high hydrophobic surfaces tend to adsorb more plasma proteins, which results in a faster blood clearance and capture by the RES.(17) Considering these facts, many strategies have been explored to avoid and/or reduce immune system activation by making the surface of NPs more hydrophilic.(17)(37) One of the most promising approaches consists in attaching hydrophilic polymers/moieties, such as polyethylene glycol (PEG), poloxamer, dextran, chitosan, poloxamine, and many

others.(17)(37) Even though this strategy may solve the clearance issues, the aggregation of small particles due to large surface area is still a concern.(37)

PEG is a hydrophilic, non-charged and relatively inert polymer that is commonly incorporated on NPs' surface, producing a stealth effect that hinders the adsorption of plasma proteins.(37)(33) However, several studies have shown that PEG-coated NPs are also capable of activate immune system after repeating injection, thus increasing their clearance.(39)(40) This phenomenon is called accelerated blood clearance (ABC) and occurs through the development of anti-PEG antibodies.(41) Other studies have also revealed the presence of detectable levels of anti-PEG antibodies in the blood of healthy patients, who have never undergone treatment with PEGylated drugs.(42)(43) These findings have raised significant concerns about the safety and efficiency of these drugs. An alternative to the use of PEG is the incorporation of zwitterion components onto the NPs' surface, such as amino acids and polybetaines. Water molecules establish a strong electrostatic bond with zwitterions, when compared to water hydrogen bonding with PEG, resulting in the higher stability of these systems. However, carboxy-based systems are pH dependent and are difficult to systematically functionalize, which limits the ability to control surface properties while maintaining biocompatibility and a corona-free character.(33)

An ideal nanocarrier for drug delivery should be able to reach, recognize, bind and deliver its load to specific disease sites, thus reducing or avoiding drug induced damage to healthy tissues.(37) To achieve this goal, targeting approaches can be used. As mentioned above, passive targeting exploits the physicochemical characteristics of the target tissues, such as the EPR effect. On the other hand, active targeting approaches mostly consist of binding targeting moieties to the surface of nanocarriers, in order to promote specific interactions with the target sites.(13) These targeting ligands are capable of specifically binding to receptors that are overexpressed by the diseased tissues or by tissues' vasculature, increasing the delivery efficiency of the drug and reducing side-effects.(13)(44)(45) The most commonly used targeting agents include small molecules, antibodies and antibody fragments, peptides, glycoproteins, vitamins, growth factors and nucleic acids.(13)(44) Small organic molecules are widely used, as they are stable and relatively easy to prepare.(37) The major drawback with these approaches is that healthy cells still express the same targeted receptors, thus ligands may not have the desired specificity.(37)(45) Considering that healthy cells greatly outnumber diseased cells, most nanocarriers will miss their target and produce side effects. One of the strategies to overcome these issues is using multiple ligands.(45) The high surface to volume ratio of nanocarriers allows the attachment of multiple targeting moieties, thereby achieving better targeting of the disease sites. The successful use of this approach requires a

homogeneous expression of the target receptor in all target cells and the exclusive binding of the targeting moiety to a receptor overexpressed only by the diseased cells.(44)

There are other targeting techniques in which physical and chemical alterations of the area of interest are exploited for the targeted delivery of drugs.(13)(46) In this case, targeting relies on the combination of bioresponsive materials with an internal or external stimulus, such as pH, reactive oxygen species (ROS), temperature, light and magnetic fields, among others.(13)(47) For instance, due to the high metabolic rate and inadequate oxygen supply, tumor extracellular space in poorly perfused regions is highly acidic, when compared with the surrounding environment of normal tissues. One possible approach is the use of pH-sensitive NPs, which are designed to be activated by low pH, in order to release the drugs into the acidic extracellular space of solid tumors.(48) Drug carriers must be capable of surviving in normal tissues, and at the same time be susceptible to degradation when the stimulus is applied. Therefore, drugs are only released in the diseased tissues, avoiding undesired systemic effects. When an external stimulus is applied to promote the degradation of carriers, its application must be strictly localized, in order to accumulate drugs only inside the area of interest.(13)

3. Types of nanoparticles

Nanocarriers can be arranged in two major groups: organic and inorganic nanocarriers. The first group includes solid lipid nanoparticles (SLNs), liposomes, dendrimers, polymeric nanoparticles (PNPs), micelles, niosomes, nanogels and virus-like nanoparticles (VLNPs); and the second group is composed by carbon nanotubes (CNTs), mesoporous silica nanoparticles (MSNs), metallic nanoparticles (MNPs), calcium phosphate nanoparticles (CaPNPs), quantum dots (QDs) and magnetic NPs.(44)(49)(16) Some organic/inorganic hybrid nanocarriers have also been developed, in order to combine the advantages of organic and inorganic materials.(44)

3.1. Organic nanoparticles

Organic nanocarriers are carbon-based nanomaterials that show high biocompatibility and improved drug loading capacity. They offer a relatively simple route for encapsulation of materials, allowing a versatile control of both morphology and chemical composition. Furthermore, their colloidal stability and relatively large size enable the incorporation and carrying of a wide range of drugs.(50)(51)

3.1.1. Lipid-based nanoparticles

<u>Liposomes</u>

Liposomes were the first nano drug delivery system to be successfully applied to the clinical practice, in 1965.(52) They are spherical lipid-based vesicles with an aqueous internal cavity

enclosed by a lipid bilayer membrane, composed of either synthetic or natural phospholipids.(13)(53)(54) These vesicles are synthesized by the hydration of dry phospholipids, in a spontaneous process, due to self-association of amphiphilic phospholipids into bilayers.(13)(54) In this process, the interactions between water molecules and the hydrophobic phosphate groups of phospholipids are responsible for the closure of the lipid bilayer, forming a sphere. The predominant physical and chemical properties of liposomes, such as permeability, charge density and steric hindrance, arise from the properties of the constituent phospholipids.(54)

Liposomes have unique advantages as drug carriers, including not only protection of drugs against enzyme degradation with low toxicity levels, but also great flexibility, biocompatibility, and biodegradability. Furthermore, liposomes are considered as non-immunogenic. Despite all these benefits, their application is limited by their short shelf life, poor stability, low encapsulation efficacy, rapid removal by RES, cell interactions or adsorption and intermembrane transfer.(53)

SLNs and nanostructured lipid carriers (NLCs)

SLNs and NLCs are colloidal carrier systems that were developed, in 1990, as alternative to liposomes, PNPs and emulsions, in order to achieve controlled drug delivery. (53)(55) SLNs are spherical particles with an average size of 50 to 1000 nm, made of a lipid matrix that is solid at human physiological temperature (37ºC).(13)(53)(54) This matrix can consist of a great range of biocompatible lipids, including mono-, di- and triglycerides, fatty acids, waxes and combinations thereof, thus minimizing the risk of acute and chronic toxicity.(13)(54)

SLNs are obtained by replacing the liquid lipid (oil) of an oil-in-water emulsion by a solid lipid, and must be stabilized with non-toxic surfactants, polymers or both, in order to form administrable emulsions.(13)(54) These surfactants avoid aggregation and stabilize the dispersion.(55) SLNs form a strongly lipophilic matrix into which drugs can be incorporated for subsequent release.(54)(55) Drug loading into the lipid matrix can be affected by several factors, including: (i) the solubility of the drug in lipid; (ii) chemical and physical properties of the lipid or lipids' mixture; (iii) crystalline characteristics of the lipid(s) at biological temperature; and (iv) the polymorphic form of the lipids used. Loading capacity can be improved by using a heterogeneous lipid mixture, since it produces an imperfect crystalline structure with larger gaps in which the drug can be incorporated. (54)

Large-scale production of SLNs can be performed in a cost-effective and relatively simple way using hot or cold high-pressure homogenization (HPH), or microemulsion techniques.(13)(56) There are other possible preparation methods, such as emulsification-solvent evaporation, solvent injection or emulsification-diffusion and ultrasonication. However, these techniques

require the use of organic solvents, which hampers mass production. HPH and microemulsion operate under high temperature, pressure and shear stress conditions that are detrimental to protein stability. This problem can be solved by using methods based on supercritical fluids, which avoid protein denaturation and degradation.(13)

SLNs combine the benefits of liquid lipid-based colloidal systems and solid systems, making them suitable for both parenteral and non-parenteral administration routes.(13)(56) They exhibit excellent tissue biocompatibility, biodegradability, composition flexibility and small size.(13) Interestingly, these carriers seem to be capable of crossing the blood brain barrier (BBB).(57)(58)(59) However, this ability differs with the molecules delivered, and also with the models used to evaluate it.(60) Later, it was found that solidification and subsequent crystallization of the lipid from the dispersed phase in SLNs lead to the expulsion of the drug from the carriers, which constitutes a serious problem of instability. This phenomenon seems to occur due to the fact that lipid molecules progressively crystallize in more stable forms, generating an increase in particle size and a decrease in the loading capacity.(61) In order to overcome the instability issues, SLNs were modified to obtain a new colloidal system in which part of the solid lipid is replaced by a liquid lipid or a mixture of liquid lipids, forming an unstructured matrix, in which the solid state of the particle is maintained at room and body temperature, the so called NLCs.(61)(62) The incorporation of the liquid lipid into the solid matrix of NPs increases the number of imperfections in the core solid matrix, thus increasing the loading capacity.(63)(62) Likewise, NLCs show greater stability, since they do not allow the recrystallization of solid lipids and, thereby, the size remains almost unchanged during storage.(61)

3.1.2. Polymeric nanoparticles

Polymers are the most commonly used materials for the construction of nanoparticle-based drug carriers, and can be divided in two main groups, according to their source: natural and synthetic polymers.(64)(65) The choice of polymer will depend on the required characteristics for the carrier.(65) The most widely used natural polymers are polysaccharides, such as chitosan, hyaluronic acid (HA), alginate, dextran and cellulose, and protein-based polymers, namely albumin, fibrin, and gelatin.(2)(66) On the other hand, synthetic polymers include PEG, polylactide (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), polycaprolactones, and polyacrylates.(2)(66)(50) Natural polymer-based NPs are highly biocompatible, non-toxic, and offer a significant improvement in efficacy and effectiveness when compared with traditional oral and intravenous drug delivery systems.(55)(65) However, they often face stability issues, such as their susceptibility to pH variations, which limits their use. Moreover, they have poor batch-to-batch reproducibility, are prone to degradation and are potentially antigenic.(55)

PNPs are colloidal systems that can be used in different formulations and are obtained through adsorption, dissolution, entrapment, encapsulation, or chemical binding of drug molecules on their surface. The drug release kinetics and its properties will solely depend on the drug trapping method and polymer structure.(65) Natural polymers are more sensitive to processing conditions, thus natural polymer-based PNPs are produced via mild technics, including ionic gelation polyelectrolyte complexation and coacervation. Instead, PNPs composed of synthetic polymers are usually prepared by more extensive methods, such as interfacial polymerization, emulsification-polymerization, emulsification-solvent evaporation, nanoprecipitation, salting out, supercritical fluids and emulsification solvent diffusion.(67)

Considering their morphology and architecture, PNPs can be presented in two forms: nanocapsules and nanospheres. Nanocapsules are vesicular systems that act as a reservoir, in which the drug is usually confined to a cavity consisting of an inner liquid core of oil or water, surrounded by a polymeric membrane (shell). The drug may also be adsorbed to the capsule surface, even though this is less common. In contrast, nanospheres are matrix systems composed by a solid mass of polymers in which the drug may be dispersed throughout the polymer matrix or adsorbed at the sphere surface.(55)(68)(65)

PNPs offer unique advantages over other carrier systems, such as biocompatibility, non-immunogenicity, non-toxicity and biodegradability, leading to a higher stability in biological fluids.(55)(67) They are extensively employed due to their high versatility and ease of formulation, and also because they allow the sustained release of the drugs and can impart stability and longer activity duration for volatile molecules.(65)(67) Furthermore, their physicochemical properties, drug release profile, and biological behavior can be modulated through the application of several polymeric materials and targeting ligands.(67) PNPs are attractive systems for intracellular and site specific delivery, and are considered ideal candidates for vaccine delivery, cancer therapy, and targeted antibiotics delivery.(55)(65)

3.1.3. Viral nanoparticles

Despite all the advances in the nanotechnology field, large-scale production of structurally homogeneous populations of NPs is still difficult to achieve. This problem can be solved by using bionanomaterials based on viruses, which allow the assembly of millions of identical NPs and their production in living cells.(69) Virus particles are typically composed of several hundreds to thousands of protein molecules that self-assemble to form a hollow scaffold packaging for the viral nucleic acid.(70) Viral nanoparticles (VNPs) are virus-based formulations that can be used as a building block for novel materials with a great range of properties.(71) They can be obtained from bacteriophages, plant or animal viruses and are broadly divided in two types: virus-based nanoparticles, that feature a modified capsid

encapsulating the virus genome; and VLNs that comprise protein components alone, which renders them non-infectious.(69)(71)(72) Viral NPs derived from plant viruses and bacteriophages are particularly advantageous, since they are less likely to be pathogenic in humans, and consequently less likely to induce undesirable side effects.(69)(71)

VNPs are dynamic, self-assembling systems that comprise regular arrays of virus coat proteins, forming a highly defined three-dimensional structure, which provides an engineering scaffold that is superior to synthetic particles.(69)(71) These particles are easily altered and functionalized by modifying the nucleic acid template that codes viral proteins prior to synthesis, and by chemically decorating the particles through addition of conjugates to specific amino acid side chains.(71)(70) VNPs offer several advantages over synthetic nanomaterials, including biocompatibility, biodegradability, and the ability to cross biological barriers and efficiently deliver the drugs into the target cells.(69)(71) Moreover, viral carriers can present a large number of targeting molecules, and also enable the control of the spacing and orientation of the ligands.(73)

3.2. Inorganic nanoparticles

Inorganic NPs cover a broad range of substances including elemental metals, metal oxides and metal salts.(74) These particles have received significant attention in preclinical development as potential diagnostic and therapeutic systems for variety of biological applications, especially in the field of oncology.(75) Inorganic nanocarriers are generally composed of a core containing the inorganic component and a shell composed mainly of organic polymers (or metals). The shell region provides a suitable substrate for the conjugation of biomacromolecules and protects the core from undesired physicochemical interactions with the surrounding biological microenvironment.(50)

3.2.1 Mesoporous silica nanoparticles

Based on their pore size, silica particles can be classified into microporous, mesoporous and macroporous particles, with pore sizes of less than 2 nm, between 2-50 nm and greater than 50 nm, respectively.(76) Silica nanoparticles with mesopores – MSNs – have received great attention over the recent years thanks to their unique structural properties, including high internal volumes, large surface areas, and uniform and tunable pore size.(76)(77)(78) These particles are composed of a high amount of narrow pores, which enable not only the adsorption of drugs and proteins into their structure, but also their controlled release.(76)(78) Pores have an opening and closing mechanism that can be controlled by diverse systems, such as polymers, nanocrystals, photoactive derivatives, and external triggers like heat, pH, light and chemicals. The release of the drug will depend on their nature, the release medium, pore size, surface functionalization and particle size and morphology.(78)

In addition to surface properties, mesoporous materials possess good biocompatibility, controllable size, and easy surface and pore functionalization, turning them into valuable candidates for several biomedical applications.(76)(77) Functionalization of mesoporous NPs is performed in order to ensure better drug delivery, higher adsorption of the drug, as well as for sustained release of drugs.(78) MSNs are internalized by the cells mainly via endocytosis, and this process can be affected by different parameters, including morphology and size of the particles, surface functionalization, and electrostatic interactions between MSNs and cell membrane.(76)

3.2.2. Metallic nanoparticles

MNPs are considered as good drug carriers and biosensors, and can be synthesized from diverse metals, although silver and gold are the most commonly used for biomedical applications.(55)

Gold nanoparticles (AuNPs)

AuNPs, also known as colloidal gold, have a size range of 3 to 150 nm and are one of the most stable metal NPs, presenting a high surface to volume ratio, as well as electrochemical, optical, magnetic (quantum-size effects) and catalytic properties.(49)(79)(80) Moreover, AuNPs have a tendency to change the color of colloidal solutions depending on their sizes.(79) As the core size increases from 1 to 100 nm, they exhibit a range of colors (e.g. brown, orange, red and purple) in aqueous solution, which make them promising agents for use in biomedical imaging and photothermal therapy applications.(80)(81)

AuNPs show excellent biocompatibility, low toxicity and are easily prepared, functionalized and dispersed in liquids.(79)(80) The most common method for the synthesis of AuNPs is chemical reduction of gold salts, in the presence of reducing agents.(49)(82) The ease of AuNPs functionalization, by producing assemblies with oligonucleotides, antibodies and proteins, along with their high surface area, provides a versatile platform for therapeutic agents, since they are able to display a dense presentation multifunctional moieties, such as drugs and targeting agents.(80)

Silver nanoparticles (AgNPs)

AgNPs are particles of silver with a size range between 1 and 100 nm that show unique physical and chemical features, including optical, electrical, thermal and biological properties, making them suitable for several applications.(82)(83)(84) They have been used as antibacterial agents in industrial, household and healthcare-related products, in medical device coatings, diagnostics, orthopedics and as drug delivery vehicles, among others.(84) Although they are commonly described as being silver, some of these particles are composed of a large percentage of silver oxide, due to their large ratio of surface to bulk silver atoms.(82) The color

of AgNPs solutions varies from light yellow to reddish brown and even black, which can provide some clues about their size and agglomeration tendency.(79)

Like AuNPs, AgNPs are typically synthesized via reduction of a salt, such as silver chloride (AgCl), silver iodide (AgI) and silver nitrate ($AgNO_3$), with a reducing agent in the presence of a colloidal stabilizer.(79)(82) However, conventional methods are expensive, as well as hazardous, due to the use of toxic substances, such as organic solvents, reducing agents, and stabilizers that are used to prevent undesired agglomeration of the colloids. These substances present significant threats, including toxicity, cytotoxicity and carcinogenicity.(84)(85)(86) More recently, other promising approaches have been developed, such as biologically-prepared AgNPs, obtained from natural resources, like plants, plant products, bacteria, fungi, algae, yeast and viruses.(84)(85) Biogenic synthesis of metal NPs can be achieved through two main mechanisms: bio-reduction, in which metal salts are chemically reduced into the elemental metal that may be stabilized by organic molecules present in the living organisms; and biosorption, which involves the binding of metal ions from an aqueous medium into the surface of the cell wall of the organisms.(87) These green chemistry techniques show high yield, solubility, and high stability. Moreover, these methods seem to be simple, rapid, non-toxic, and reliable.(85)

3.2.3. Paramagnetic nanoparticles

Paramagnetism is a type of magnetism in which atoms with one or more unpaired electrons are attracted by an externally applied magnetic field. The application of this field induces a magnetic moment that is reverted to the ground state once the field is removed. This transition is called relaxation and is described by T1 and T2 relaxation time parameters that represent the return of the longitudinal and transverse magnetization to the equilibrium state, respectively. The relaxation rate depends on the paramagnetism of the material and when the applied magnetic field strength is held constant, the T1 and T2 relaxation times are distinct not only for different tissue types, but also between diseased and healthy tissues.(88)

Magnetic NPs are a class of particulate materials of less than 100 nm in size, composed by magnetic elements, including cobalt, nickel, iron and their respective oxides, such as magnetite, maghemite, cobalt ferrite and chromium dioxide.(55) They exhibit remarkable properties, including high field irreversibility, high saturation field, superparamagnetism, extra anisotropy contributions, and shifted loops after field cooling, which arise from surface and finite-size effects that dominate the magnetic behavior of individual NPs.(89) These particles have been used for a wide range of applications in areas like medicine, biology, and materials science, due to their biocompatibility with low toxicity, easy surface modification, and magnetic properties.(90)

The main uses of magnetic NPs in biomedicine include analytical applications, in which particles are used as magnetic carriers in separation processes, as biosensors for detecting molecular recognition events, and as contrast agents for magnetic resonance imaging (MRI), and therapeutic approaches, such as drug delivery and hyperthermia during cancer therapy.(90)(91) Magnetic NPs can bind to a great number of biological molecules, such as proteins, enzymes, antibodies, and nucleotides, and direct them to specific tissues or organs through the application of an external magnetic field.(90) The process of drug localization using magnetic delivery systems is based on the competition between the forces exerted on the particles by the blood compartment an the magnetic forces produced by the magnet. Through magnetic targeting, NPs remain fixed at the local site while the drugs are released, acting locally and reducing side effects, as well as the dosage required.(89) The biggest advantage of magnetic applications is the precision afforded by the technique.(49)

In spite of all the advantages, the use of magnetic NPs as drug carriers has some drawbacks. These particles are more likely to be cleared by macrophages or RES and tend to aggregate owing to strong magnetic dipole-dipole interactions between particles trying to reduce the energy associate with the high surface area to volume ratio. Furthermore, non-coated magnetic NPs are chemically reactive, and are oxidized when they come in contact with air, resulting in a loss of their magnetization. Thus, like other NPs, these particles need to be stabilized by modification with biocompatible surfactants, polymers, and oxide compounds with functional groups.(90)

4. Toxicity

The toxicity of NPs is highly determined by their physical and chemical properties, such as their size, shape, specific surface area, surface charge, catalytic activity, and the presence or absence of a shell and active groups on the surface.(92) These properties influence how they interact with cells and, thus, their overall potential toxicity. Understanding these interactions can lead to the development of safer NPs.(93)

Particle size plays critical role on nanotoxicity. The surface area and volume ratio of NPs increase exponentially with size reduction, increasing the available surface area to interact with cellular components like nucleic acids, proteins, fatty acids, and carbohydrates.(93)(94) Smaller particles are more likely to enter the cell, causing cellular damage.(93) Size-dependent toxicity was observed in both *in vitro* and *in vivo* studies using gold(95)(96) and silver(97) NPs, in which smaller size particles resulted in high cytotoxicity. However, the same is not true for all types of NPs. Jiang et al.(98) used titanium dioxide (TiO_2) NPs within a size range of 4 to 195 nm to compare the amount of ROS production per surface area. The results showed that the 30 nm NPs produced the highest ROS activity and that this activity dramatically decreased

as size decreased from 30 to 10 nm. Therefore, the relationship between nanoparticle properties and nanotoxicity seems to be complex, depending on the combination of several factors.

Surface chemistry and charge are other two key parameters that determine the NPs interactions with biological systems. Small NPs have an increased number of atoms and crystal lattice defects on their surface, which enhances the surface energy and reactivity. This energy can be released by the formation of radicals such as ROS, causing DNA and protein damages. Moreover, the dissolution of toxic ions from the surface of NPs, including Zn^{2+}, Cu^{2+} and Ag^{2+}, can also produce serious organelle damage and cellular dysfunction. The occurrence of these deleterious effects will depend on the composition nature of the nanomaterials used.(94) Particle surface charge, on the other hand, may affect the cellular uptake of particles, as well as the way they interact with organelles and biomolecules, thereby influencing cytotoxicity. NPs with higher surface charges produce greater toxicity effects, since positively charged NPs are easily be internalized by the cells due to electrostatic interactions with negatively charged cell membrane glycoproteins.(92)(93) These particles also have the ability to interact with other negatively charged molecules such as DNA, causing irreversible damages.(93) Another important issue that must be taken into account is that proteins from NPs corona can also affect surface properties of NPs, altering their surface charge, aggregation characteristics, and/or hydrodynamic diameter. Furthermore, the adsorption of proteins on the NPs surface leads to changes in their conformational structures, which may decrease or completely inhibit their functional activities, causing disturbances in several biological processes.(92)

Shape also affects toxicity, in particular the aspect ratio, which has a direct impact on detrimental effects produced by one dimensional materials (e.g. nanowires, nanorods and nanotubes), changing the fate of the cell-uptake and biodistribution of NPs. The impact of the aspect ratio on toxicity is difficult to determine due to interference factors generated form nanofabrication processes.(94) Significant efforts have been made in order to develop suitable *in vitro* and *in vivo* toxicity testing assays, or to adapt previous developed methods that are used for bulky materials, to assess nanomaterial-induced toxicity. However, the validation of new techniques remains challenging, since most nanomaterials are insoluble and have a tendency to aggregate, which influence exposure doses by interfering with optical measurements and inducing nonlinear dose-response relationships. Moreover, currently available methods are unable to detect effects at low doses. Therefore, high-throughput tools are required so that NPs toxicity can clearly be link to their physicochemical properties and unique challenges of nanomaterial research could be adequately addressed.(15)

5. Nanoparticles for protein-based therapies

Over the past three decades, several nanomedicines have been developed and commercially approved for clinical use, with many more being currently under clinical investigation. They were primarily developed for drugs which have low aqueous solubility and high toxicity, in order to reduce their side effects while increasing the pharmacokinetic properties.(99) Nanoparticle formulations of small-molecules, such as doxorubicin (Doxil® and Myocet®), daunorubicin (DaunoXome®), paclitaxel (Abraxane®), and amphotericin B (Ambisome®) have shown considerable success, paving the way for the exploration of nanoparticle technologies for protein delivery. Even though conventional small-molecular drugs continue to dominate the overall pharmaceutical market, protein therapeutics offer the advantages of increased circulation half-lives, higher specificity, greater activity, and less toxicity.(4)(100) Therapeutic proteins include monoclonal antibodies, cytokines, tissue growth factors, vaccines and gene transfer products that are used for the prevention and treatment of many diseases.(101)

Nanomedicines' development faces numerous challenges, making the transition of nanotechnology from the bench to the market difficult. Some of these issues are related to physicochemical characterization, biocompatibility and nanotoxicology evaluation, pharmacokinetics and pharmacodynamics assessment, process control, as well as scale-reproducibility.(102) The lack of standard protocols for the characterization of nanomedicines at physicochemical and physiological/biological levels has often limited the efforts of many researches to evaluate the potential toxicity of nanodrugs in the early stages of testing, resulting in failures in late-phase clinical trials. A closer cooperation among regulatory agencies is mandatory to simplify and/or shorten the approval process for nano-based medicines.(99)

5.1. Approved protein-based nanotherapies

FDA has adopted the definitions of "nanotechnology", "nanoscale", "nanomaterial", and other related terms from the engineering of materials field.(102) Thus, nanoscale materials are defined as nanomaterials (i.e. materials used in the manufacture of nanomedicine, aditives, etc.), and final products (nanomedicines) with a particle size of 1 to 100 nm.(100) Currently, from a list of more than 50 nanotechnology based-products approved for clinical practice in the USA, 17 are protein-based therapies, which are presented on **Table 1**. Almost all of them are PEGylated proteins.

The first approved PEG-protein conjugate was Adagen® (Enzon Pharmaceuticals Inc., 1990), a PEGylated form of adenosine deaminase (ADA) used to treat severe combined immunodeficiency (SCID), which is characterized by an inherited deficiency in the ADA protein.(103) The deficiency in this protein will lead to the accumulation of adenosine and 2-deoxyadenosine, resulting in metabolic disorders related to the functions of lymphocytes.(104)

Table 1 - FDA approved protein-based nanotherapies.(100)(105)(106)

Product Name	Marketing-authorization holder	Active Pharmaceutical Ingredients	Formulation	Indication(s)	Approval Year
Adagen®	Leadiant Biosciences Inc.	Pegademase bovine	PEG-protein conjugate	SCID	1990
Oncaspar®	Enzon Pharmaceuticals Inc.	Pegaspargase	PEG-enzyme conjugate	Acute lymphoblastic leukemia	1994
Copaxone®	Teva Pharms USA	Glatiramer acetate	Copolymer of L-glutamate, L-alanine, L-lysine and L-tyrosine	Multiple sclerosis	1996
Curosurf®	Chiesi USA	Poractant alfa	Liposome	Respiratory distress syndrome	1999
Ontak®	Eisai Inc.	Denileukin diftitox	Fusion protein	Cutaneous T-cell lymphoma	1999
PegIntron®	Merck	Pegylated IFN α2b	PEG-protein conjugate	Hepatitis C	2001
Neulasta®	Amgen Inc.	Pegfilgrastim	PEG-protein conjugate	Chemotherapy-induced neutropenia	2002
Pegasys®	Genentech	Pegylated IFN α2a	PEG-protein conjugate	Hepatitis B and C	2002
Zevalin®	Acrotech Biopharma	90Y-ibritumomab tiuxetan	Radiolabeled antibody	Non-Hodgkin's lymphoma	2002
Somavert®	Pfizer	Pegvisomant	PEG-protein conjugate	Acromegaly	2003
Eligard®	Tolmar Pharmaceuticals	Leuprolide acetate	PLGA nanoparticle	Prostate cancer	2004
Mircera®	Vifor Pharma	Methoxy polyethylene glycol-epoetin beta	PEG-protein conjugate	Chronic kidney disease-associated anemia	2007
Cimzia®	UCB Inc	Certolizumab pegol	PEG-protein conjugate	Crohn's disease, rheumatoid arthritis, psoriatic arthritis, plaque psoriasis ankylosing spondylitis	2008
Krystexxa®	Horizon Pharma	Pegloticase	PEG-protein conjugate	Chronic gout	2010
Plegridy®	Biogen	Pegylated interferon β-1a (IFN β-1a)	PEG-protein conjugate	Multiple sclerosis	2014
Adynovate®	Takeda	Antihemophilic factor (recombinant), pegylated	PEG-protein conjugate	Hemophilia	2015
Rebinyn®	Novo Nordisk Inc.	Coagulation factor IX (recombinant), glycopegylated	PEG-glyco-protein conjugate	Hemophilia B	2017

The success achieved by this therapy laid the foundations for a number of different PEGylated protein therapeutics being approved by the FDA, including PEGylated interferon-α2b (IFN-α2b) and interferon- α2a (IFN- α2a), which are used for the treatment of hepatitis B and C and are commercialized as PegIntron® (Merck, 2001) and Pegasys® (Genentech, 2002), respectively.(103) These therapies were followed by others like Cimzia® (UCB Inc., 2008), used for the treatment of autoimmune conditions, such as rheumatoid arthritis and Crohn's disease, Krystexxa® (Horizon Pharma, 2010) for chronic gout, and Plegridy® (Biogen, 2014), indicated for multiple sclerosis. Despite PEGylated proteins representing the great majority of FDA approved protein-based nanotherapies, there are other approved formulations, including protein copolymers (Copaxone®, 1996), liposomes (Curosurf®, 1999), fusion proteins (Ontak®, 1999) and PLGA NPs (Eligard®,2004).(100)(105)

In contrast with the FDA, the EMA working group established its own definition of nanomedicines as systems designed with the purpose of clinical applications, with at least one component at nano-scale size, resulting in definable specific properties which are related to the specific nanotechnology application and characteristics for the intended use (route of administration, dose), while being associated with the expected clinical advantages of nanoengineering (e.g. preferential organ/tissue distribution).(107) In the European Union (EU), protein-based nanotherapeutic products authorized for marketing by the EMA (**Table 2**) are exclusively PEGylated proteins, with the exception of Zevalin® (Bayer Pharma, 2004), a radiolabeled antibody used for the treatment of non-Hodgkin lymphoma.(100)(108) All EU marketed products were first approved by the FDA and have the same commercial name as in the USA. The only exception is Adynovi®, the Adynovate® European counterpart that was approved by EMA in 2018 for treatment and prophylaxis of bleeding in patients 12 years and above with hemophilia A. Krystexxa® (Crealta Pharmaceuticals Ireland Limited) was approved by the EMA in 2013, having however been withdrawn from use in the EU since 2016.(108)

It is also important to notice that not all protein nanotherapies commercialized in European countries were approved under the centralized authorization procedure, either because they were authorized before EMA's creation or because they were not in the scope of this authorization procedure. In centralized procedure, pharmaceutical companies submit a single-marketing authorization application to EMA that once approved is valid in all EU member states, as well as in the European Economic Area (EEA) countries of Iceland, Liechtenstein and Norway.(108) Copaxone® and Eligard® are examples of non-approved EMA therapies that are commercialized in Portugal under national authorization procedures.(108)(109)

Table 2 - EMA approved protein-based nanotherapies.(100)(108)

Product Name	Marketing-authorization holder	Active Pharmaceutical Ingredients	Formulation	Indication(s)	Approval Year
PegIntron®	Merck Sharp & Dohme B. V.	Pegylated IFN α -2b	PEG-protein conjugate	Chronic hepatitis C	2000
Pegasys®	Roche Registration GmbH	Pegylated IFN α -2a	PEG-protein conjugate	Chronic hepatitis B and C	2002
Neulasta®	Amgen Europe B. V.	Pegfilgrastim	PEG-protein conjugate	Chemotherapy-induced neutropenia	2002
Somavert®	Pfizer Europe MA EEIG	Pegvisomant	PEG-protein conjugate	Acromegaly	2002
Zevalin®	Bayer Pharma	90Y-ibritumomab tiuxetan	Radiolabeled antibody	Non-Hodgkin's lymphoma	2004
Mircera®	Roche Registration GmbH	Methoxy polyethylene glycol-epoetin beta	PEG-protein conjugate	Anemia associated with chronic kidney disease (CKD)	2007
Cimzia®	UCB Pharma SA	Certolizumab pegol	PEG-protein conjugate	Rheumatoid arthritis	2009
Plegridy®	Biogen Netherlands B. V.	Pegylated IFN β-1a	PEG-protein conjugate	Multiple sclerosis	2014
Oncaspar®	Les Laboratoires Servier	Pegaspargase	PEG-protein conjugate	Acute lymphoblastic leukemia	2016
Adynovi®	Baxalta Innovations GmbH	Rurioctocog alfa pegol	PEG-protein conjugate	Hemophilia A	2018

Compared to conventional formulations, most of the nanotherapies approved to date have shown reduced toxicity rather than improved efficacy. As matter of fact, several nanodrugs have not survived clinical development, since they failed to demonstrate a significant improvement in efficacy and improved toxicity could be achieved with other drugs or nanoformulations.(106)

5.2. Regulatory framework

Nanotechnology has presented a considerable growth in recent years, and all countries are increasing their investments in research and development in this field.(99) Although the list of nanomedicines available in the market is quite extensive, the lack of specific regulatory guidelines for the development and characterization of these nanomaterials end up hampering their clinical potential.(99)(110) In fact, the methods that are employed for testing the safety, toxicity, biocompatibility, or efficacy of these products are the same as the ones used for conventional dosage forms.(110) From the regulatory point of view, the active pharmaceutical ingredient is the one that dictates the characteristics that should be analyzed to apply for commercial approval. For instance, protein or antibody-based nanomedicines must meet the same requirements defined for biological medicinal products and for new chemical entities.(110)(111)

FDA advises that evaluations of safety, effectiveness, public health impact, or regulatory status of nanotechnology products should consider any unique properties and behaviors that the application of this technology may impart.(102) The evaluation of formulation properties of nanomedicines should comprise not only the analysis of physicochemical properties of the nanoproduct itself, but also of their composing elements and relative proportions, as well as the assessment of quality and manufacturing process used to obtain these materials. Once this first evaluation is completed, pharmacokinetic characterization and toxicity profile should also be assessed.(100)

In the EU, nanotherapeutic products are currently regulated under a conventional regulatory framework which has proven itself to be suitable for the evaluation and lifecycle management of these products.(112)(113) However, considering their complexity, additional expert evaluations are needed to ensure the quality, safety, and efficacy of these therapeutics. Several actions have been taken in order to provide regulatory guidance and assistance for the development of new high-quality, effective and safe nanotherapeutics.(112) European and other international experts, as well as medical regulatory agencies of the EU, US, Japan and Canada have recognized the need for sharing and discussing the global academic, industrial and regulatory experience and perspectives in the field of nanomedicines in order to harmonize the requirements on the different regions.(112) From this common conscience resulted many international reflections, hosted by these agencies, aiming to define the characteristics of medicines based on nanotechnology, as well as to discuss and share information on relevant on-going guidelines and scientific and legislative initiatives in the various regions.(113) These actions aim to ensure that regulatory science continues evolving alongside with the advances in the understanding of nanotechnology, and also to direct the development of new nanomedicines toward timely and effective clinical translations.(112)(113)

5.3. Investigational protein-based nanotherapies

Over the past years, many nanosystems have been investigated for the efficient delivery of therapeutic proteins, with bone morphogenetic protein-2 (BMP-2), insulin, erythropoietin (EPO) and recombinant human growth factors being among the most studied ones.(2) Oral insulin delivery has received special attention and several studies have been carried out in order to evaluate the efficacy of insulin-loaded NPs in the management of diabetes mellitus(114)(115)(116) In 2010, Sonaje and colleagues(114) constructed a pH-sensitive nanoparticle system composed of chitosan and poly(y-glutamic acid) for oral delivery of insulin. To avoid NPs disintegration and degradation of insulin in the stomach, NPs were freeze-dried and filled in enteric-coated capsules. The results showed an enhanced intestinal absorption of insulin and a prolonged reduction in blood glucose levels. More recently, Zhang et al.(116) developed innovative NPs for oral and liver-targeted delivery of insulin by using enterohepatic

circulation of bile acids. These particles were obtained from a combination of cholic acid, modified chitosan and hydroxypropyl methylcellulose phthalate (HPMCP), and demonstrated to protect loaded insulin from denaturation and degradation in the gastrointestinal (GI) tract. This approach could not only increase the oral pharmaceutical availability of loaded insulin to 30%, but could also maintain the hypoglycemic effect for more than 24 hours.

Recombinant human erythropoietin – epoetin-α – is a glycosylated protein that is prescribed to regulate the red blood cell count in the treatment of anemia induced by several conditions, including renal dysfunction, chemotherapy, bone marrow transplantation, and AIDS. Furthermore, EPO is a tissue protective agent that can reduce inflammation, inhibit apoptosis and promote angiogenesis. However, intravenous injection of EPO requires frequent administration, due to its short half-live (approximately 8,5 hours after intravenous injection), which can have a negative impact in the patients' compliance.(117) Fayed et al.(118) have demonstrated that the administration of EPO-loaded PLGA NPs to a mouse model may significantly prolong its activity, allowing for more than 2-week activity after a single injection of a double EPO dose. A previous study using a neonatal rat model of unilateral ischemic stroke, had already shown that PLGA NPs containing EPO present neuroprotective and beneficial effects after brain ischemia, with the required doses of EPO being 10 times lower when compared with free administration of EPO.(119) Later, the effect of chitosan-tripolyphosphate nanoparticles (CS-TPP NPs) loaded with EPO on an immunoglobulin A nephropathy (IgAN) rat model was evaluated. The results showed that the levels of blood urea nitrogen (BUN) and creatinine were significantly lower in the group treated with these particles, whereas the hemoglobin level has increased in this group. These changes were maintained for less than 1 week following the end of the treatment with CS-TPP-EPO.(120)

Nasal and pulmonary administration of proteins have received remarkable attention, since they exhibit low proteolytic activity when compared with oral route, are highly vascularized and have large absorptive surfaces, especially in the lungs, resulting in improved absorption. However, the large size of proteins, as well as their proteolytic instability may compromise their absorption by these mucosal surfaces.(67) Thus, nanoparticle-based nasal and pulmonary delivery of protein therapeutics provides another promising area of investigation for improving protein bioavailability to treat either local or systemic diseases. In a study using bleomycin-induced pulmonary fibrosis model rats, msFGFR2c loaded biomimetic phosphorylcholine-chitosan nanoparticles (PCCs-NPs) were obtained via ionic gelation. The orotracheal administration of the NPs resulted in a significant antifibrotic efficacy, with reduction in inflammatory cytokines, remarkable attenuation of lung fibrosis score and collagen deposition, and a significant increase in survival rate. These results strongly suggest that PCCs-NPs might be a promising nanocarrier for pulmonary protein delivery.(121) NPs have also been tested as

delivery systems for nasal vaccines, since they can improve antigen delivery to the immune cells and, at the same time, limit their mucosal clearance.(122)

Therapeutic protein delivery to the retina has also emerged as a useful but challenging approach for the treatment of several prevalent degenerative diseases, such as age-related macular degeneration, diabetic retinopathy and retinitis pigmentosa. Since formulations used for topical application are rapidly cleared and blood-retinal barrier reduces the efficacy of systemic administered drugs, repeated bolus intravitreal injections remains the standard route of administration. However, they present higher risk of drug overdose, inflammation and cataracts.(123) Nanomedicine technology offers a great platform for designing minimally or even non-invasive systems to deliver drugs to the retina in a sustained manner. By using NPs as delivery vehicles for ophthalmic agents, it is possible to improve the solubility of poorly water-soluble drugs, target the drug to the retina, enhance the cellular uptake of the drug, aid the transport of the drug through biological barriers, increase residence time, and protect the drug from degradation.(124) In a recent study, Delplace et al.(123) developed a bioengineered intravitreal hyaluronan and methylcellulose hydrogel for sustained, local therapeutic protein delivery to the retina, using ciliary neurotrophic factor (CNTF), a protein known for its neuroprotective effect on the retina. In order to control the release of CNTF, it was recombinantly expressed as a fusion protein with Src homology 3 (SH3) domain (CNTF-SH3), while the hydrogel was modified with an SH3 binding peptide, thus allowing reversible binding of the fusion protein to the gel matrix. The structure, stability, bioactivity and controlled release of CNTF-SH3 were first investigated *in vitro* and then in a mouse model. The results showed successful affinity-based delivery of CNTF-SH3 to the mouse retina, and demonstrated the safety of the delivery system, paving the way towards new intravitreal protein strategies.

There is still a final research area, advanced tissue engineering, which is widely explored in clinical trials using protein therapies, particularly regarding its application to bone tissue regeneration. Recent efforts have been focused on the use of natural or synthetic matrices which combine biodegradability with the properties of protein delivery vehicles, allowing for implanted cell actions and enhanced tissue regeneration.(125) BMP-2, a growth factor that induces osteoblast differentiation and promotes bone regenerations, has been extensively investigated for this purpose.(126) BMP-2 loaded NPs demonstrate to be capable of significantly enhancing osteogenic differentiation, being a promising method for bone regeneration applications.(125)(127) MSNs have also been widely applied in bone tissue engineering, for instance in the construction of scaffolds, due to their highly specific surface areas, ease of chemical modification, large pore volumes, controllable particle size, and favorable biocompatibility.(126)(128) Zhou and colleagues(126), covalently grafted a BMP-2 derived peptide on the surface of MSNs via an aminosilane linker, and simultaneously loaded

dexamethasone (DEX) into the channels of the particles, obtaining a nanoparticulate osteogenic delivery system (DEX@MSNs-pep). This system promoted *in vitro* osteogenic differentiation of bone mesenchymal stem cells (BMSCs) in terms of the levels of alkaline phosphatase (ALP) activity, calcium deposition, and expression of bone-related protein. An effective osteoblast differentiation and bone regeneration were also observed in vivo, after 3 week intramuscular implantation in rats.

IV. Concluding Remarks

Over the past decades, peptides and proteins have received considerable attention as potential therapeutic agents for the treatment of several diseases due to their great effectiveness, high specificity and biocompatibility. However, their systemic instability has compromised the efficient delivery of these molecules to target sites, thereby limiting their clinical application. The development of nanoformulations for the sustained delivery of proteins and peptides represented a huge step towards the development of protein-based therapies. Despite that, there are still several remaining challenges that need to be overcome in order to obtain safe, stable and efficient protein-loaded nanoconstructions which can be submitted to clinical trials. Each application requires the formulation of an adequate carrier, adapted to its specific needs in terms of size, composition, surface functionalization, drug compatibility and targeting properties, which renders the process expensive and difficult to scale for mass industry production.

Toxicity assessment still has a long way to go so that suitable *in vitro* and *in vivo* assays can be developed and validated, in order to obtain more sensitive reports that can clearly associate the physicochemical properties of these materials to their toxicological profile. Furthermore, regulatory framework also need to evolve alongside the advances in the nanotechnology field, establishing specific guidelines to support the development and characterization of new formulations. Despite all the issues that still need to be addressed, protein-loaded NPs hold great promise as new therapeutic agents for targeted therapies, increasing protein bioavailability, controlling their release and efficiently targeting organs and tissues.

V. References

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